HISTONE DEACETYLASE INHIBITION AFFECTS SODIUM IODIDE SYMPORTER (NIS) EXPRESSION AND INDUCES ^{131}I CYTOTOXICITY IN ANAPLASTIC THYROID CANCER CELLS.

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Abstract

Background: Anaplastic thyroid cancers (ATC) represent only 1-2% of all thyroid tumors, but they account for up to 50% of the mortality. Treatment of differentiated thyroid carcinomas is well standardized and the use of radioiodine represents an essential step; in contrast, there is no standardized therapeutic approach for anaplastic tumors and their prognosis is poor. The resistance of anaplastic thyroid cancer to radioiodine treatment is principally due to the absence of expression of the sodium iodide symporter (NIS), mainly due to epigenetic silencing. The acetylation status of histones is involved in the epigenetic control of gene expression and is usually disrupted in advanced thyroid cancer. Histone deacetylase inhibitors have been demonstrated as potent anticancer drugs with several different effects on cell viability and differentiation.

Methods: Stabilized anaplastic thyroid cancer cell lines (BHT-101 and CAL-62) and primary cultures from patients who underwent thyroidectomy for anaplastic thyroid cancer were treated with the histone deacetylase inhibitor LBH589. After treatment, we evaluated the expression and function of NIS. Gene expression was evaluated by real-time PCR (RT-PCR); NIS promoter activity was determined with a luciferase reporter assay; and protein expression was assessed through immunofluorescence. We tested the protein function by ^{125}I uptake and efflux experiments; finally the cytotoxic effect of ^{131}I was determined with a clonogenic assay.

Results: Our results demonstrate that treatment with LBH589 leads to NIS RNA expression as shown by RT-PCR and luciferase assay, and to protein expression as determined by immunofluorescence *in vitro* and by immunohistochemistry in xenograft tumors. Moreover, ^{125}I uptake and efflux experiments show the correct protein function and iodine retention, that translate into cytotoxicity effects, as demonstrated by a clonogenic assay with ^{131}I .

Conclusions: This study supplies a new potential strategy for the treatment of ATC by modifying gene expression with the aim of inducing responsiveness towards radioiodine therapy.

Introduction

Anaplastic thyroid carcinoma (ATC), despite its low incidence, is one of the most lethal human malignancies, accounting for about 50% of all thyroid carcinoma deaths (1, 2). Patients with ATC have a median survival of 3-5 months (2) even in the absence of metastasis at the time of diagnosis. At present, therapies available for ATC include chemotherapy, radiotherapy and surgery, but the outcomes are very poor; hence, there is a need for new therapeutic strategies.

Epigenetic alterations, which in the "epigenetic progenitor model" (3) are considered to be equally if not more important than mutational events for cancerogenesis, led to the concept of epigenetic therapy. Indeed, there is a strong basis for the use of "epigenetic modifier drugs" to modulate the epigenome of cancer cells to a status resembling the one seen in normal cells, thereby leading to tumor cell death or restoration of normal differentiation (4).

The presence of the sodium iodide symporter (NIS) is both a diagnostic and a therapeutic tool in the management of thyroid cancer due to its ability of mediating iodide uptake (5). Unfortunately, approximately 10–20% of thyroid tumors comprising metastatic differentiated thyroid carcinomas, as well as poorly differentiated and anaplastic cancers (6), fail to uptake radioiodine and are unable to concentrate enough ^{131}I for effective cytotoxic therapy; tumors with reduced or absent NIS activity are therefore generally associated with a poor prognosis.

Deacetylase inhibitors (DCI) are compounds that are able to alter the chromatin acetylation status, modulating the transcription of about 2% of genes involved in growth, differentiation and apoptosis (7). NIS gene silencing has been related to histone acetylation. The use of DCI like trichostatin A (TSA), sodium butyrate (NaB) and valproic acid restored NIS expression in human thyroid cancer cell lines (8-10). Moreover, increased NIS expression was demonstrated in non-thyroid cell lines after treatment with TSA, NaB (10) and LBH589 (11). As far as ATC is concerned, we have reported elsewhere that valproic acid increased NIS expression in poorly differentiated thyroid cancer cell lines (9), but the effect of the drug in ATC is limited to mRNA expression without any consequence on iodine uptake. Panobinostat or LBH589 is a potent pan-deacetylase inhibitor with

anti-cancer activity as demonstrated *in vitro* against classical Hodgkin's lymphoma (cHl), and in clinical phase I and phase II trials in patients with relapsed cHl (12). We recently demonstrated that LBH589 affects histone acetylation and has potent cytotoxic and anti-invasive activities in *in vitro* and *in vivo* ATC (13,14).

The aim of the present work is the evaluation of LBH589 ability to induce NIS expression and activity in ATC in order to obtain significant radioiodine uptake and sensitivity .

Materials and methods

Cell lines and reagents

Anaplastic thyroid carcinoma (BHT-101 and CAL-62) cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Both cell lines were maintained at 37°C, in 5% CO_2 and 95% humidity, in D-MEM-F12 (Invitrogen, Groningen, The Netherlands), with 100 IU/ml penicillin and 100 µg/ml streptomycin added, supplemented with 10% heat inactivated FCS (Euroclone, Wetherby, West York, UK). LBH589 was provided by Novartis Pharma AG (Basel, Switzerland), prepared as a 5mM stock solution in DMSO and stored at -80°C.

Primary culture establishment

Anaplastic thyroid carcinoma samples were obtained from 7 patients undergoing thyroidectomy for ATC (cultures were labeled CPa-g). Samples obtained from two healthy thyroids were used as controls (labeled CPS1 and CPS2). Informed consent for the study was obtained from each patient. Tissues were obtained under sterile conditions and processed within 24 hours of surgery. Samples were washed three times in saline containing penicillin/streptomycin and nystatin and then minced in D-MEM-F12 and incubated overnight with 0.25% trypsin at 4°C. After this, enzymatic digestion was performed using 0.35% collagenase (Sigma, Milano, Italy) at 37°C for 60 min. Cells were filtered through double layers of sterile gauze and washed twice with D-MEM-F12 plus 20% FCS. Cells were cultured in complete medium containing 100 IU/ml penicillin and 100 µg/ml streptomycin, 50µg/ml gentamicin, 2.5µg/ml amphotericin B and 20% FCS.

Primary cultures were tested for cytokeratins by immunofluorescence (monoclonal Anti-pan Cytokeratin Clone C-11, Sigma, Milano, Italy); and for *thyroglobulin (Tg), Thyroid Transcription Factor 1 (TTF-1)* and *NIS* expression by RT-PCR. All the cultures showed complete concordance with the original tumors or normal thyroid tissues (Table 1).

Gene expression evaluation with RT- PCR

Cells ($1x10^6$) were seeded in $75cm^2$ flasks and treated with increasing doses of LBH589 (0 - 50 nM). After 24 hours of treatment, total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK). DNase I was added to remove remaining genomic DNA. 1 µg of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (BioRad Laboratories, Inc.), following the manufacturer's protocol. Primers (Table 2) were designed using Beacon Designer 5.0 software according to parameters outlined in the BioRad iCycler Manual. The specificity of the primers was confirmed by BLAST analysis. RT-PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 µl containing 12.5 µl IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 1 µl of each primer at 10 nM concentration, and 5 µl of the previously reverse-transcribed cDNA template. The protocol for each primer set was optimized using seven serial 5X dilutions of template cDNA obtained from cells in basal conditions. The protocol used was as follows: denaturation (95°C for 5 min), and 40 cycles of amplification (95°C for 15 sec, 60°C for 30 sec). A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. For each sample all the reactions were carried out at least in triplicate. Results were normalized using the geometric mean for three different housekeeping genes (β-actin, β2-microglobulin and L13A) and expressed as relative expression fold versus the lower level of expression.

NIS Promoter Luciferase Assay

Cells (2.5×10^5) were seeded in 6-well plates. 24 hours after seeding, cells were transfected with 1 µg of NIS-luc plasmid/well using Lipofectin Reagent (Invitrogen Ltd, Paisley, UK). The plasmid, donated by Prof. G. Damante (Dipartimento di Scienze & Tecnologie Biomediche, Policlinico Universitario di Udine, Udine) contained 2.2 kb of 5' genomic sequence of the NIS promoter linked to the luciferase (LUC) gene as reporter (15). After 24 hours, cells were treated with LBH589 (10-

25 nM) for a further 24 hours. At completion luciferase activity was assayed with a Luciferase Assay System (Promega Corporation, Madison, WI, USA).

NIS immunofluorescence microscopy

Cells (3 x 10^3) were seeded in 96-well plates. 24 hours after seeding cells were treated with LBH589 (25 and 50 nM) for 48 hours. After treatment cells were fixed in acetone/methanol (1:1) at -20 °C for 20 minutes, washed with PBS containing 0.5% Triton X-100, 0.05% NaN_3 and 1% BSA and incubated overnight with monoclonal anti-NIS antibody (1:1000) (FP5A, Thermo Fisher Scientific, CA, USA) in PBS at 4°C. The cells were then washed three times with PBS containing 0.5% Triton and 0.05% NaN_3 for 10 minutes followed by detection with an anti-mouse Cy3-conjugated secondary antibody (1:1000) (GE Healthcare Europe, GmbH, Milan, Italy) in PBS plus 0.5% Triton and 0.05% NaN_3 for 2 hours and the nuclei were counterstained with Hoechst 33258 (500 ng/ml in DMSO) in PBS. Cells were washed twice with distilled water and dried at 37°C.

^{125}I uptake and retention by cells

Cells (2 x 10^4) were seeded in 24-well plates and incubated with LBH589 for 48 hours. ^{125}I uptake was assayed as reported elsewhere (9). Briefly, after aspirating drug-containing medium, cells were washed with 1 ml HBSS (Invitrogen, Groningen, The Netherlands). ^{125}I uptake was initiated by adding 0.5 ml HBSS containing 2 µCi carrier-free Na^{125}I (PerkinElmer Life and Analytical Sciences, Inc., Boston, USA) and 30 µM NaI. Samples were then incubated 30 min at 37°C. At completion the radioactive medium was removed, the cells were then washed twice with ice-cold HBSS and solubilized with 1 ml of absolute ethanol for 20 min, after which cell-associated iodide was measured in a γ-counter (PerkinElmer Life and Analytical Sciences, Inc., Boston, USA). To avoid any unspecific iodide uptake, control cells were pre-incubated with 300 µM $KClO_4$ for 30 min and then treated with ^{125}I, as described above.

Iodide efflux was studied by incubating cells for 30 min at 37°C in HBSS incubation buffer plus 2 µCi carrier-free Na^{125}I (PerkinElmer Life and Analytical Sciences, Inc., Boston, USA) and 30 µM NaI. The medium was then replaced every 5 min with fresh HBSS, for 30 minutes. Afterwards,

cells were solubilized with 1 ml absolute ethanol. Radioactivity was counted in both the medium and the cells. Total radioactivity at the beginning of the efflux (100%) was calculated by adding the radioactivity of all the media to those of the cells. The percentage of activity remaining in the cells at different times was obtained by subtracting the activity at each time from that of the total radioactivity at the beginning (16).

Measured radioactivity was normalized by cell number and total activity in both the uptake and the efflux experiments.

^{131}I cytotoxicity

$1x10^2$ cells were seeded into 24-well plates and left 48 hours to attach. 24 hours after treatment with 25 nM LBH589, a clonogenic assay was performed. Briefly, drug-containing medium was discarded and cells were washed twice in PBS; the medium was then replaced with D-MEM-F12 (Invitrogen, Groningen, The Netherlands) in the presence or absence of 20μCi ^{131}I (GE Healthcare S.r.l., Italy) for 6 hours. At the end of the treatment the radioactive medium was discarded, cells were washed and left in regular culture medium for the time corresponding to six doublings. Finally, cells that had formed colonies were fixed in methanol and stained with crystal violet. Control and ^{131}I treated colonies were counted (17).

Xenograft studies

FFPE samples of tumors generated in SCID mice subcutaneously inoculated with CAL-62 cells and treated for 21 days with LBH589 (controls, n = 5; 20 mg/kg LBH589, n = 5), as described elsewhere (13), were assessed for the presence of NIS protein, using automated immunohistochemistry slide processing platforms (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA and Bond Max Leica, Germany) and a primary monoclonal antibody against human NIS (Clone FP5A Thermo Fisher Scientific, Fremont CA, USA).

NIS was evaluated by one of the authors (SA) using the following semi-quantitative evaluation: 1) negative: no cells were stained; 2) positive: cells showed membrane, and/or sub-nuclear, and/or cytoplasm stain.

Statistical analysis

Data are expressed throughout the text as means ± SD, calculated from at least three different experiments. Comparison between groups was performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $p < 0.05$.

Results

LBH589 effect on NIS expression

To investigate the effect of LBH589 on *NIS* expression, we performed RT-PCR on both stabilized and primary cell cultures. In BHT-101 and CAL-62 cells, LBH589 stimulated *NIS* expression in a dose dependent-manner (Fig.1, panels A and B); these results were further confirmed by the activation of a *NIS* promoter luciferase construct in both cell lines (Fig.1, panels C and D). LBH589 was also effective in inducing *NIS* expression in primary cell cultures, that did not express *NIS* in basal conditions (Table 1). In fact, we found *NIS* mRNA expression in all the cell lines treated with LBH589, albeit at different levels (Fig.3 panel A). As far as other thyroid related genes (i.e. *TTF-1*, *Thyroglobulin, TSH-R* and *PAX8*) are concerned, no modulation of the expression was observed (data not shown) after LBH589 treatment.

NIS protein and function

Immunofluorescence was performed on both stabilized cell lines and primary cultures to assess NIS protein expression. As shown in Fig.2, panels A and B, no NIS protein was present in either BHT-101 or CAL-62 under basal conditions, but treatment with LBH589 induced the expression of the protein. This result was evident in all the primary cultures as shown in Fig. 3, panel B. NIS was correctly localized also on membranes of tumors treated with 25 nM LBH589 (5/5); all control cases were negative (0/5). A representative control case (panels A and B) and a treated tumor (panels C and D) are shown in Fig.4.

^{125}I uptake experiments confirmed not only the presence but also the function of the protein. In both stabilized cell lines we observed a dose dependent increase of iodide uptake (Fig.2, panel C and D). In primary cultures we chose a single concentration of 25 nM for the experiments of ^{125}I uptake and we found ^{125}I uptake restoration after LBH589 treatment in every cell culture (Fig.3, panel C). Iodine uptake was specifically dependent on NIS because it was blocked in response to treatment with $KClO_4$, as shown in Fig.3 panel D. To evaluate the ability of ATC cells to retain iodide, we performed efflux studies, where the cells were repeatedly incubated with HBSS. As reported in

Fig.5, iodide retention was observed in both CAL-62 (83% retention after 5 min, $t_{1/2}$ = 19 min) and CPa (79% retention after 5 min, $t_{1/2}$ = 17 min), suggesting that iodide is adequately retained by ATC cells after treatment with 25 nM LBH589.

Iodide-induced cytotoxicity

Iodide uptake is not enough to deduce a possible cytotoxic effect of radioiodine on cells after LBH589 treatment. Therefore, to demonstrate radioiodine cytotoxic effects, a clonogenic assay with ^{131}I was performed in a stabilized cell line (CAL-62) and in a primary cell culture (CPa). As shown in Fig.6, we observed a reduction in LBH589-treated colonies forming cells after ^{131}I treatment in both cell lines; in fact the number of colonies was significantly smaller in cells treated with 25 nM LBH589 compared to untreated controls (CAL-62: $p < 0.05$; CPa $p < 0.01$).

Discussion

The absence of the sodium iodide symporter (NIS), along with other iodide-handling genes and specific thyroid transcription factors (18), characterizes anaplastic thyroid carcinomas, and results in resistance to radioiodine treatment. Since epigenetic modifications contribute to the silencing of thyroid-specific genes in advanced cancers, drugs modifying the epigenome are considered a promising tool to obtain a functional NIS (19). Recently it has been shown that global levels of acetylated histones are modified in thyroid cancer tissues and that lower levels of acetylated H3K18 are associated with tumor progression (20). In the present study we demonstrate that LBH589 induces NIS expression promoting the cytotoxic activity of radioiodine in anaplastic thyroid cancer.

In fact, LBH589 induced *NIS* mRNA expression in a dose dependent manner in both stabilized cell lines and in all the primary cultures obtained from patients who underwent thyroidectomy for anaplastic thyroid cancer. The ability of other DCI to act on NIS expression in anaplastic thyroid cancer cells has already been reported (10, 21, 22). However, some previous studies showed the effect of DCI in ARO and /or BHP cells (10, 21), which are not of thyroid origin (23). The induction of *NIS* gene expression resulted in an increase in NIS protein expression, which was clearly demonstrated in all the cells we tested. In addition, in our *in vivo* model, NIS protein correctly localized on the membranes of tumors from LBH589-treated mice. Valproic acid as well as other drugs, such as 5'-Aza-2'-deoxycytydine and retinoic acid were able to restore *NIS* mRNA and protein expression in thyroid cancer cell lines, but this treatment was unable to restore protein function (9, 24).

Here we also show that LBH589 activated *NIS* promoter in cells transfected with a construct containing the NIS promoter fused to luciferase. The *NIS* promoter can be activated by several thyroid-specific transcription factors (6,10). Even if various additional genes encoding tumor-suppressors or proteins involved in thyroid hormone synthesis are epigenetically silenced in thyroid tumors (25, 26), we could not find a significant modulation of the expression of the other studied genes (*TTF-1*, *TG, PAX8* and *TSH-R*) in any of the cell lines, consistent with other studies (10). The

observation that DCI modulate *NIS* expression in non-thyroid cells (that are not expressing thyroid-restricted transcription factors) such as the human breast cancer cell line MCF-7 (10, 11), strengthens the concept that LBH589 may directly affect the *NIS* promoter, even though the established mechanism has not been investigated.

We also demonstrated that LBH589 allowed iodide uptake and retention in both immortalized cultures of confirmed thyroid origin (23) and in primary cultures. The release of iodide that we observed was similar to that of cells stably expressing NIS (27, 28). Our data finally demonstrated that LBH589 was effective in inducing cytotoxic effects mediated by radioiodine in anaplastic thyroid cancer cells. The ability of LBH589 to express a functional symporter that allows radioiodine treatment appears extremely relevant and opens up therapeutic perspectives for undifferentiated thyroid tumors.

Because of its rarity and rapidly fatal course, ATC is difficult to investigate at both the clinical and the experimental levels (29). Nevertheless, testing drugs on primary cultures may be considered an interesting way to design new strategies for chemo- and gene therapy (30), with the aim of increasing the effectiveness of treatment in each single patient. We were able to set up primary cultures for studying the effects of LBH589. We constantly observed an increase in NIS expression, albeit at variable levels. These differences could be explained by a variable sensitivity to the drug due to the well-known heterogeneity of ATC. Moreover, the variability in iodide uptake in each primary culture is somehow similar to the great inter- and even intra-patient spectrum in radioiodine kinetics that are observed in patients treated with standard ^{131}I doses (31).

In conclusion, the present work suggests that histone deacetylase inhibitor LBH589 is an effective differentiating agent conferring to ATC cell lines and primary cultures radioiodine sensitivity. If confirmed, it provides a basis for clinical studies aimed at demonstrating an improvement of radioiodine uptake in patients with ATC.

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Author Disclosure Statement

The authors have nothing to disclose.

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Figure Legends

Figure 1. ***LBH589 effect on the expression of the NIS gene in BHT-101 (panel A) and CAL-62 (panel B) cell lines.*** mRNA expression was evaluated with RT-PCR. Results are normalized for three different housekeeping genes (β-actin, β2-microglobulin and L13A) and expressed as relative expression fold *vs* lower level of expression (*, P<0.05; *** P<0.001). ***LBH589 effect on transcriptional activity of the NIS promoter in BHT-101 (panel C) and CAL-62 (panel D).*** Cells were transiently transfected with a reporter plasmid carrying the luciferase gene under the control of the promoter of NIS. The luciferase activity was assayed after LBH589 treatment; data are expressed as mean ± SD; n=3 Significance *vs* lower level of activity * P< 0.05

Figure 2. ***LBH589 effect on NIS protein expression in BHT-101 (panel A) and CAL-62 (panel B) cell lines***. Immunofluorescence for NIS was performed on cells in basal condition and after treatment with 25 and 50 nM LBH589. Magnification *400x*. ***LBH589 effect on iodide uptake in BHT-101 (panel C) and CAL-62 (panel D) cell lines***. ^{125}I uptake in cells treated with 5-50 nM LBH589 was evaluated as described; data are expressed as mean ± SD; n=3. Significance *vs* untreated cells (0) was: *P<0.05; **P< 0.01; ***P< 0.001.

Figure 3. ***LBH589 effect on NIS gene expression in primary cultures (panel A).*** mRNA expression was evaluated with RT-PCR. For each primary culture, results were first normalized for three different housekeeping genes (β-actin, β2-microglobulin and L13A), and then expressed as relative expression fold *vs* CPS1 and 2 (normal controls for primary cultures). ***LBH589 effect on NIS protein expression in primary cultures (panel B).*** Immunofluorescence for NIS was performed on cells in basal conditions and after treatment with 25 nM LBH589. Magnification *400x*. ***LBH589 effect on iodide uptake in primary cultures.*** ^{125}I uptake in cells treated with 25 nM LBH589 was evaluated as described; iodine uptake is expressed as relative expression fold *vs* CPS1 and 2 (iodide uptake for CPS=47,000 cpm/10^6 cells ; for CPS2=43,000 cpm/10^6 cells). Data are expressed as mean ± SD; n=3 ***(panel C).*** ^{125}I uptake in cells treated with $KClO_4$ is expressed as

relative expression fold *vs* CPS1 and 2, and represented as mean ± SD of ^{125}I uptake experiments of all the primary cultures ***(panel D).***

Figure 4. Immunohistochemistry of NIS in a sample from an untreated tumor xenograft (***panel A***: H&E; ***panel B***: IHC) and in tumor xenograft treated with LBH589 (panel C: H&E; panel D: IHC). Panel B, inset: salivary gland tissue used as positive control.

Figure 5. ^{125} Iodide retention in a stabilized cell line (CAL-62) and a primary culture (CPa) chosen as representative samples. Total radioactivity at the beginning of the efflux (100%) was calculated by adding the radioactivity of all media to those of the cells. The percentage of activity remaining in the cells at different times was obtained by subtracting the activity in each timed sample from that of the total radioactivity at the beginning. Results are normalized to total activity and cell number; experiments were performed in triplicate.

Figure 6. ***LBH589 effect on ^{131}I cytotoxicity***. ^{131}I cytotoxicity was evaluated through a colony forming assay as described; data are represented as number of colonies before (^{131}I -) or after (^{131}I +) the addition of 20 μM ^{131}I to untreated cells (basal) or cells treated with 25 nM LBH589. Data are expressed as mean ± SD; n=3. A representative stabilized cell line (CAL-62, ***panel A***) and a primary culture (CPa, ***panel B***) are shown. Significance of cells treated with 25 nM LBH589 vs cells untreated with the deacetylase inhibitor: $^{*}p < 0.05$; $^{**}p < 0.01$.

Table 1: Main characteristics of patients, original tissues and primary cultures

Primary Cell Cultures					Subjects			Original Tissue				
	Citokeratins	***Thyro globulin***	***TTF1***	***NIS***	***Age, yr***	***Sex***	***pTNM***	***Histology***	***Citokeratins***	***Thyro globulin***	***TTF1***	***NIS***
CPa	Neg	Neg	Neg	Neg	70	Female	pT4a N0 M1	ATC	Neg	Neg	Neg	Neg
CPb	Neg	Neg	Neg	Neg	58	Male	pT4a N1b M0	ATC	Neg	Neg	Neg	Neg
CPc	Neg	Neg	Neg	Neg	70	Male	pT4a N0 M0	ATC	Neg	Neg	Neg	Neg
CPd	Neg	Neg	Neg	Neg	65	Male	pT4a N0 M0	ATC	Neg	Neg	Neg	Neg
Cpe	Neg	Neg	Neg	Neg	58	Female	pT4a N0 M1	ATC	Neg	Neg	Neg	Neg
CPf	Neg	Neg	Neg	Neg	81	Female	pT4a N1a M1	ATC	Neg	Neg	Neg	Neg
CPg	Neg	Neg	Neg	Neg	59	Male	pT4a N0 M0	ATC	Neg	Neg	Neg	Neg
CPS1	Pos	Pos	Pos	Pos	61	Female	----------	Normal	Pos	Pos	Pos	Pos
CPS2	Pos	Pos	Pos	Pos	65	Male	----------	Normal	Pos	Pos	Pos	Pos

Table 2. Primers for real time PCR

Gene	***Primers***
TTF-1	Sense: 5’- CTC GCT CGC TCA CCC GTT GGC -3’ Antisense: 5’-GTC GTG TGC TTT GGA CTC ATC G -3’
PAX8	Sense: 5’- ATC AAT GGG CTC CTG GGC ATC-3’ Antisense: 5’-GAG GTG GTG CTG GCT GAA GG -3’
TSH-R	Sense: 5’- ACC CTG ATG CCC TCA AAG AGC-3’ Antisense: 5’- GCT TCA GTG TCA AGG TTT CAT TGC -3’
Thyroglobulin	Sense: 5’- TCT AAC CGA TGC TCA CCT CTT CTG - 3’ Antisense: 5’- AGA TGA TGG CAC CTC CTT GAA CC - 3’
NIS	Sense: 5'- CTG CCC CAG ACC AGT ACA TGC C -3' Antisense: 5'- TGA CGG TGA AGG AGC CCT GAA G -3'
β-Actin	Sense: 5’- GCG AGA AGA TGA CCC AGA TC- 3’ Antisense: 5’- GGA TAG CAC AGC CTG GAT AG-3’
β2-Microglobulin	Sense: 5’-AGA TGA GTA TGC CTG CCG TGT G-3’ Antisense: 5’-TCA ACC CTC CAT GAT GCT GCT TAC-3’
L13A	Sense: 5’-GCA AGC GGA TGA ACA CCA ACC-3’ Antisense: 5’-TTG AGG GCA GCA GGA ACC AC-3’

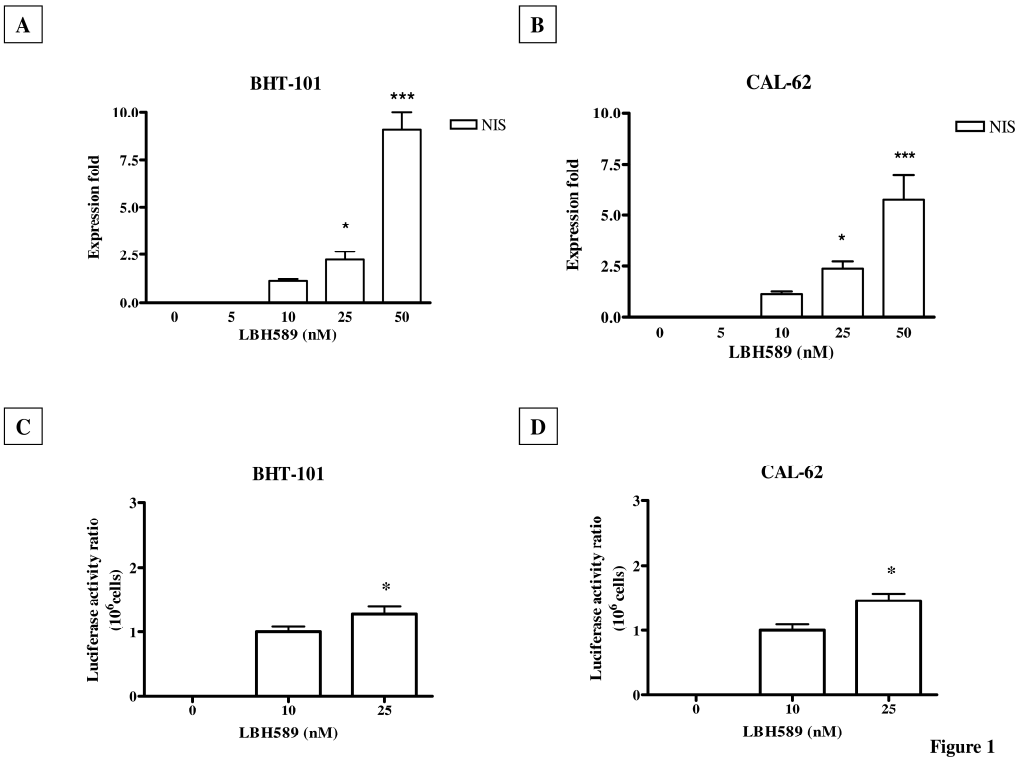

A
BHT-101
NIS
Expression fold
10.0
7.5
5.0
2.5
0.0
0
5
10
25
50
LBH589 (nM)
*
***
B
CAL-62
NIS
Expression fold
10.0
7.5
5.0
2.5
0.0
0
5
10
25
50
LBH589 (nM)
*
***
C
BHT-101
Luciferase activity ratio
(10^6cells)
3
2
1
0
0
10
25
LBH589 (nM)
*
D
CAL-62
Luciferase activity ratio
(10^6cells)
3
2
1
0
0
10
25
LBH589 (nM)
*

Figure 1

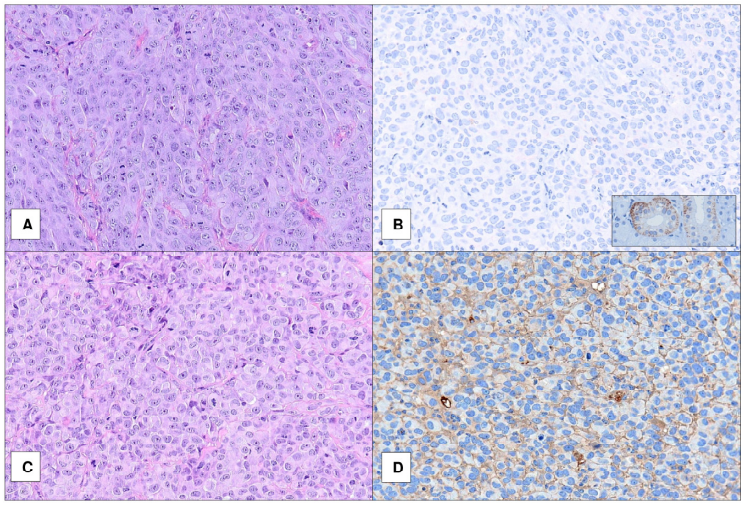
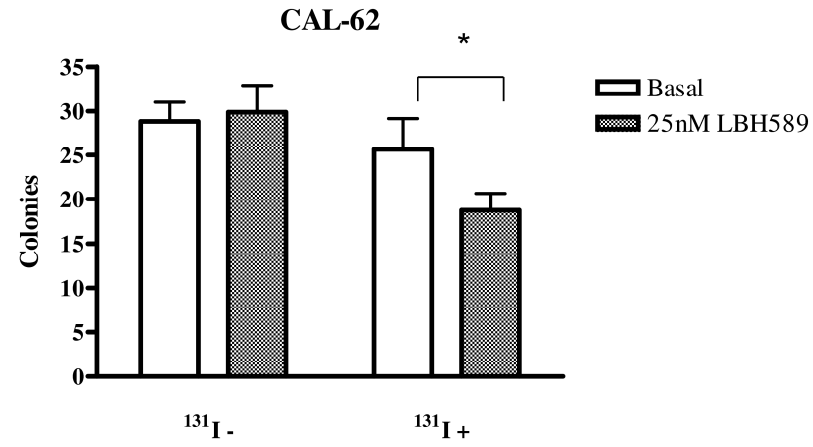


Figure 4


CAL-62
*
Colonies
35
30
25
20
15
10
5
0
131I -
131I +
Basal
25nM LBH589

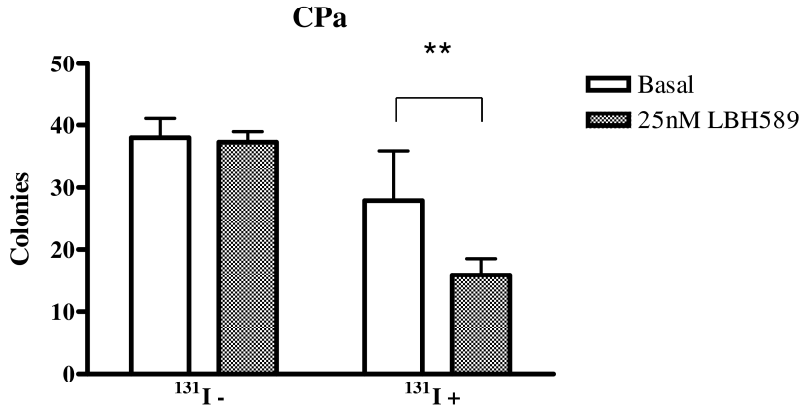

CPa
**
Colonies
50
40
30
20
10
0
131I -
131I +
Basal
25nM LBH589

Figure 6